C5L2, a Nonsignaling C5A Binding Protein†

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ABSTRACT: C5a anaphylatoxin, a potent inflammatory mediator, is known to act through a specific G protein coupled receptor. However, some of the complex effects of C5a in vivo may not be explained solely by the deletion of the known receptor. Here, we show that an orphan receptor, identified as C5L2, is a high affinity C5a binding protein. Unlike the previously described C5aR, C5L2 is obligately uncoupled from heterotrimeric G proteins, in part by virtue of an amino acid alteration in the so-called DRY sequence at the end of the third transmembrane segement. Both human and murine C5L2 bear a leucine for arginine replacement at this site. C5L2, when transfected into several cell types, is weakly phosphorylated in transfected cells following binding of C5a but does not induce significant activation of MAP kinases, mediate calcium flux, or stimulate chemotaxis. Bone marrow cells from wild type respond robustly to C5a with induction and suppression of a number of inflammation related genes. In contrast, C5a receptor deficient mice, which bear C5L2 alone, do not respond to C5a with changes in gene transcription by microarray analyses. Biophysical properties of the C5L2, including slow ligand on and off rates, absence of internalization, and relatively high affinity for the C5a des Arg metabolite, suggest that this receptor may serve to modulate C5a biological functions in vivo. Finally, in contrast to previous reports, we find absolutely no interaction of C5L2 with other anaphylatoxins C3a and C4a.

The complement anaphylatoxins are prominent mediators of host defense and inflammation (*1*). Ancestral components of the innate immune system, these peptides are released following activation of the proteolytic cascade of complement activation. Many diverse biological activities are mediated via complement proteins other than the anaphylatoxins, including lysis of foreign cells or virally infected cells by the membrane attack complex, C5b-9, phagocytosis following opsonization by C3 fragments, and regulation of the adaptive immune response through C3 fragments (*2*, *3*). Apart from its critical role in host defense, the C5a molecule has been implicated as a major mediator in systemic inflammatory response syndrome, asthma, reperfusion injury, myocardial infarction, rheumatoid arthritis, and other autoimmune diseases (*4*). More recently, several groups have linked the C3a molecule to experimental models of asthma in mice, rats, and guinea pigs, with correlative data for specific activation of complement in antigen-challenged human asthmatics (*5*−*8*).

The anaphylatoxins function on a wide variety of bone marrow derived cells via coupling to G protein coupled receptors (*1*, *9*). Additionally, expression of receptors for C3a and C5a on parenchymal cells in the lung, liver, smooth muscle, and endothelial cells serve as yet uncharacterized functions (*10*, *11*). To more fully understand the function of C3a and C5a, we generated mice deficient in the C3a and C5a receptors (*5*, *12*). Because of some of the complex effects of C3a and C5a in vivo (i.e., biological activity of C3a des Arg in mononuclear cells (*13*) and potential antiinflammatory and pro-inflammatory effects of C5a in systemic inflammation or sepsis, respectively (*14*, *15*)), we have suspected that additional receptors for the anaphylatoxins might exist. Recently, an orphan receptor that localizes to the same region of chromosome 19 as the C5a and formyl peptide receptors (*16*) was identified as C5L2[1] (C5a-like receptor 2) and was shown to be present on immature (but not mature) dendritic cells (*17*). Because of the pattern of tyrosines and acidic N-terminal residues, which we have shown to be a major feature of the extracellular C5a binding domain (*18*), we cloned C5L2 from both mouse and man. Here, we present evidence that C5L2 is actually a high affinity receptor for both C5a and C5a des Arg. We detail the expression of the receptor at the protein and mRNA levels, biophysical binding properties, molecular basis of uncoupling from G proteins, phosphorylation following agonist binding, lack of internatization, and absence of interaction with the anaphylatoxins C3a and C4a.

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[1] Abbreviations: C5L2, C5a-like receptor 2; MAP kinase, mitogen activated protein kinase; TM, transmembrane.

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EXPERIMENTAL PROCEDURES

Preparation and Purification of Recombinant Human C5a and C5a des Arg. Human recombinant C5a was purchased from Sigma (St. Louis, MO). Human C5a and C5a des Arg cDNAs were prepared by RT-PCR from human lung based upon previously published sequence data (*19*) (accession number NM 001735). The PCR primers included a *BamH*I site at the 5′ end and two in-frame stop codons at the 3′ end. The PCR product was inserted into the expression vector pQE-80L (Qiagen, Valencia, CA) such that a 12 amino acid N-terminal tag containing an initiating methionine and a 6X histidine metal-binding domain was in frame with the C5a or C5a des Arg coding sequence. Recombinant peptides were produced using the QIA express system, purified by affinity chromatography, and renatured as previously described (*20*). The C5a hexapeptide analogue Phe-Lys-Ala-dCha-Cha-dArg (*21*) was synthesized commercially (New England Peptide).

Molecular Characterization of the Murine C5L2 Gene. The coding sequence of human C5L2 was obtained by RT-PCR using human brain cDNA (Clontech) as a template and the following primers: 5′-ATGGGGAACGATTCTGTC-3′ (sense) and 5′-CTACACCTCCATCTCCGA-3′ (antisense). A murine genomic library (Stratagene) was screened using ^{32}P-labeled cDNA corresponding to human C5L2 and yielded a ~15 kb clone encoding mouse C5L2. Genomic analysis was performed using DNA from FVB/NJ mice, restricted with *Eco* RI, *Eco* RV, *Stu*I, or *Hind*III. Southern blots were hybridized with ^{32}P-labeled mouse C5L2 cDNA.

For Northern blot analysis, total RNA was prepared from tissues derived from BALB/c mice using RNAqueous (Ambion). RNA (5 μg per lane) was electrophoresed on 1.1% denaturing agarose gels, transferred to nylon membranes, and hybridized with ^{32}P-labeled mouse C5L2 or C5aR cDNA probes. Human RNA blots were purchased from Clontech and hybridized with human C5L2 or C5aR probes in the same manner as the mouse blots.

Expression Plasmids and Transfection. The coding sequences of human C5aR and C5L2 were cloned into pcDNA3.1 to include a nine amino acid C-terminal tag corresponding to the C-terminus of bovine rhodopsin (C9 tag) to facilitate recognition by the antibody 1D4 (National Cell Culture Center, Minneapolis, MN). The plasmid encoding the human C5L2 variant, in which leucine132 was mutated to arginine, was generated using the QuickChange method (Stratagene). All constructs were confirmed by sequencing.

HEK293T cells (ATCC, CRL11554) were transiently transfected with human C5aR, C5L2, or C3aR with or without Gα16 using calcium phosphate and used for further experiments 36−48 h later (*16*). The murine pre-B-lymphoma line, L1.2, was stably transfected by electroporation (*22*), and stable clones were selected with 0.8 mg/mL G418.

Binding Studies. Binding experiments were performed with L1.2 and HEK293T cells transfected as described above or with human peripheral blood neutrophils. 2×10^5 293T cells or 1×10^6 L1.2 cells or PMNs were incubated with 0.1 nM ^{125} I-C5a or 0.5 nM ^{125}I-C3a (Dupont/NEN Life Science Products) and 0−300 nM unlabeled C5a (Sigma-Aldrich), C3a or C4a (Advanced Research Technologies), 0−13.2 μM C5a des Arg, or 0−1 mM C5a hexapeptide analogue for 30 min at 37 °C in binding buffer (20 mM HEPES, pH 7.4, 125 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 0.5 mM glucose, and 0.2% BSA). Cells were washed by centrifugation, and bound ligand was quantitated by γ counting. All points were determined in duplicate, and at least three independent experiments were performed for each. Results are expressed as the % maximal binding (±SEM), and data were analyzed using PRISM software testing one- or two-site models of competition binding.

Kinetics of binding on stably transfected L1.2 cells were determined by incubating cells in binding buffer containing 0.02% NaN_3, at 1×10^7/mL with 0.1 nM ^{125}I-C5a at 0 °C. Duplicate aliquots were removed as a function of time for up to 60 min, filtered on 1% polyethyleneimine-soaked glass fiber filters (GF/F, Whatman), and washed 3× with ice-cold binding buffer. The remaining samples were divided into equal parts, 250 nM C5a was added to one and buffer to the other, and samples were removed with time for an additional 60 min. All points were determined in duplicate, and at least three independent experiments were performed for each. Results were expressed as the mean cpm bound (±SEM), and data were analyzed using PRISM software.

Preparation of Antisera to C5L2 and Flow Cytometric Analyses. A synthetic peptide corresponding to the predicted N-terminal extracellular sequence of human C5L2 (MGNDSVSYEYGDYSDLSDRPVDC) was coupled to KLH and utilized for production of rabbit antiserum (Alpha Diagnostics). For flow cytometric analyses, cells (either L1.2 transfectants or PMNs) were suspended in PBS containing 2% fetal bovine serum at 1×10^7/mL. Aliquots of 100 μL were incubated with 10 μg/mL rabbit anti-human C5L2, mouse anti-human C5aR (Serotec) (*23*), or irrelevant controls for 1 h at 0 °C. Cells were washed and incubated with FITC-conjugated goat anti-rabbit or PE-conjugated goat anti-mouse IgG, respectively, and fixed in 4% paraformaldehyde in PBS for flow cytometry. Analyses were performed utilizing a dual laser cytometer (Facs-Scan), and data were analyzed with Cellquest software (Becton Dickinson).

Internalization. Quantitation of receptor internalization was performed utilizing human PMNs (*1*), incubated with the synthetic C5a hexapeptide agonist at the concentrations indicated for 5 min at 37 °C. Samples were transferred to 0 °C, and receptor remaining on the cell surface was quantified by indirect immunofluorescence and flow cytometry as described above.

Calcium Mobilization by C5aR, C5L2, and C5L2(L132R). 293T cells were transiently transfected with plasmids encoding human C5aR, C5L2, or C5L2(L132R) with or without plasmid encoding the G protein subunit Gα16 that was shown to greatly facilitate C5a-mediated signal transduction (*24*). Cells were harvested at 48 h and incubated with the indicator dye Fura-2-AM (Molecular Probes) for 1 h at 37 °C in 20 mM HEPES, pH 7.4, 125 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 0.5 mM glucose, and 0.2% BSA. Cells were washed twice and resuspended in the same buffer at 2×10^6/mL. Changes in intracellular calcium concentration in response to C5a or C3a were determined by monitoring the fluorescence emission at 510 nm with excitation at 340 and 380 nm as a function of time. Responses were quantified as the peak of the ratio of 340/380 nm wavelengths.

Receptor Phosphorylation. L1.2 transfectants were washed in phosphate-free DMEM and suspended at 3×10^7/mL with

1 mCi carrier-free ^{32}P-labeled orthophosphoric acid (Dupont/NEN Life Science Products) for 3 h at 37 °C. Cells were then stimulated with 200 nM C5a for 5 min, quenched with 9 mL of ice-cold PBS, centrifuged, and lysed in 500 μL of ice-cold lysis buffer (20 mM Tris-HCl pH 7.5, 1% *n*-dodecyl-β-D-maltoside (Anatrace), 1 mM EGTA, 50 mM NaF, 50 μM Na_3VO_4, 20 mM *p*-nitrophenyl phosphate, 5 mM benzamide, and 1 mM PMSF). The lysates were cleared by centrifugation at 18 000*g* at 4 °C for 10 min. Receptors were immunoprecipitated with 1D4 antibody covalently cross-linked to protein A Sepharose beads (Sigma-Aldrich) for 1 h at 4 °C with gentle agitation. Samples were washed, eluted with SDS sample buffer under reducing conditions for 10 min at 60 °C, and applied to 10% SDS polyacrylamide gels. Following electrophoresis, gels were fixed, dried, and exposed to BioMax MR film (Kodak).

Analysis of MAP Kinase Activation. Phosphorylation of MAP kinase was investigated using L1.2 cells stably transfected as described above. Cells were washed twice with serum free RPMI and preincubated at 2×10^6/mL for 30 min at 37 °C with gentle agitation. Cells were then treated with 0 or 200 nM C5a for 0−30 min. Cells were immediately collected in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 50 mM NaF, 50 μM Na_3VO_4, 20 mM *p*-nitrophenyl phosphate, 5 mM benzamide, and 1 mM PMSF) and frozen in liquid nitrogen. After three cycles of freezing and thawing, lysates were centrifuged at 2500*g* for 10 min at 4 °C. The supernatants were further centrifuged at 97 000*g* for 1 h at 4 °C and subjected to Western blot analysis. Following electrophoresis on 10% polyacrylamide gels, proteins were transferred to nitrocellulose and stained with rabbit anti-active-MAPK antibody (Promega) as described by the supplier. Blots were incubated with peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch), and signals were detected by ECL Plus (Amersham Pharmacia Biotec) using BioMax MR film (Kodak).

Microarray Analyses. Mouse bone marrow cells were prepared from the long bones of wild type and C5aR deficient Balb/c mice. Cells were gently aspirated into RPMI containing 2% FCS using a 25 g needle. Cells were dispersed by passing through a 21 g needle, centrifuged at 400*g* for 10 min, and resuspended in RPMI at 1×10^7 cells/mL. Aliquots of 1 mL were transferred to six well-dishes and preincubated for 30 min at 37 °C in 5% CO_2. Cells were then stimulated with 0 or 50 nM C5a in PBS containing 0.1% BSA (low endotoxin) for 4 h. Total RNA was extracted using RNA aqueous (Ambion) and subjected to microarray analyses (Harvard Medical School Microarray Facility).

GeneChips were analyzed using MAS 5.0 software (Affymetrix) to create .chp files, which were copied to Microsoft Excel and saved as tab delimited text files. Tab delimited text files were analyzed using GeneSpring software (Silicon Genetics) and normalized as follows.

The 50th percentile of all measurements was used as a positive control for each sample; each measurement for each gene was divided by this synthetic positive control, assuming that this was at least 10. The bottom 10th percentile was used as a test for correct background subtraction. This was never less than the negative of the synthetic positive control. Each gene was normalized to itself by making a synthetic positive control for that gene and dividing all measurements for that gene by this positive control, assuming it was at least 0.01. This synthetic control was the median of the gene's expression values over all the samples. Last, normalized values below 0 were set to 0.

Analysis of the fold change in gene expression was calculated using GeneSpring (Silicon Genetics). First, data file restrictions were placed on the text files to include only genes with an absolute call of present (P). Then experimental samples were compared using the fold change analysis tool in GeneSpring. To determine the differential expression of genes, data file restrictions were placed on the text files to create lists of genes with the absolute call of present and absent. The gene lists were then compared using the Venn diagram tool of GeneSpring. Annotation tables of gene lists were created using the batch analysis tool of NetAffyx (Affymetrix.com). Text files of probe set IDs were imported into NetAffyx, and resulting annotation tables were downloaded and saved as Excel files.

RESULTS

Molecular Cloning of C5L2 from Mouse and Man. We confirmed the sequence of human C5L2, cloned by RT-PCR from human brain cDNA. Using this cDNA as a probe, we then isolated a genomic clone from a mouse 129Sv DNA library. Figure 1A shows the aligned sequences, which possess 61.3% identity in the deduced protein and 68.9% identity in the nucleotide sequences. Notably, the unusual replacement of the essential arginine residue in the DRY sequence following the third transmembrane segment is conserved in mouse as in man as a leucyl residue. The G protein recognition sequence in the third intracellular loop (between TM5 and TM6) is also shorter than exists in the C5aR by several cationic residues. Further, the N-terminus retains the tyrosines flanked by acidic amino acids, which we have demonstrated to be modified in the C5a receptor by sulfation, and constitutes the major C5a docking site (*18*).

As shown in Figure 1B, the C5L2 gene exists as a single copy in the mouse genome, and under the stringency conditions utilized, does not cross hybridize with the C5aR gene (not shown). To compare the expression patterns of the mRNA for human and murine C5L2 with the C5aR, we analyzed multiple tissues and cells for both receptors. We note virtually congruent expression of both C5aR and C5L2 in all tissues studied, with apparent message size heterogeneity for both human and murine C5L2 (Figure 1C). As the genomic clone encodes the entire coding sequence in a single exon, it appears that alternative splicing in untranslated regions accounts for this heterogeneity. While the bone marrow and peripheral blood leukocytes were the most abundant sources of message, both C5aR and C5L2 are expressed in other organs as well.

Expression of C5L2 and C5aR in Vivo. Because the major message abundance for both receptors occurs in leukocytes, we sought to ascertain whether they were coexpressed on the same cells and at what relative abundance. To accomplish this, we prepared an affinity-purified polyclonal antibody against a synthetic peptide corresponding to the extracellular N-terminal sequence of human C5L2. Figure 1D shows the results of a flow cytometric experiment demonstrating the specificity of the antisera in recognizing C5L2 but not the C5a receptor in the stable transfectants. We then assessed

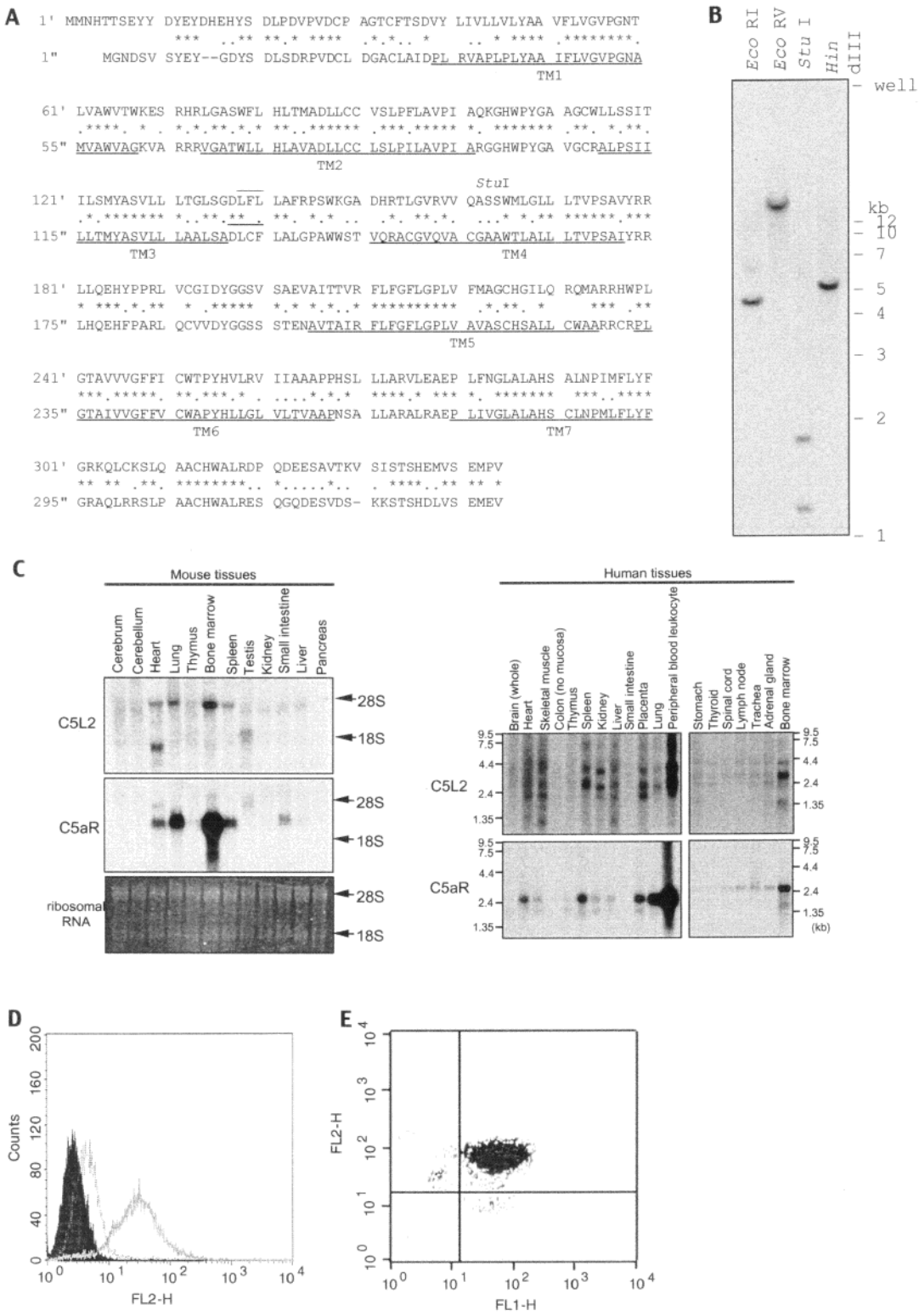

FIGURE 1: Cloning of the murine C5L2 gene. (A) Deduced amino acid sequence (single-letter amino acid notation) of mouse C5L2 (top line) aligned with the sequence of human C5L2 (bottom line). Asterisks indicate identical amino acids found in both sequences, and periods indicate chemically similar amino acids. Hyphens have been introduced to maximize sequence homology. Underlines show putative transmembrane segments with TM1-TM7 denotations. The overlines represent the sequences corresponding to the DRF sequence found in the C5a receptor. The recognition site for *Stu*I used in the Southern blot is indicated above the sequence. (B) Southern blot analysis. Mouse genomic DNA was digested with restriction endonucleases as indicated above the panel and hybridized with a cDNA probe corresponding to the full-length coding region of mouse C5L2. Only *Stu*I cuts the deduced open-reading frame of mouse C5L2. (C) Northern blot analysis of C5L2 and C5a receptors in the mouse (left panels). Five μg of total RNA from murine tissues was electrophoresed on a gel, transferred to a nylon membrane, and hybridized sequentially with mouse C5L2 (upper panel) or mouse C5a receptor (middle panel) probes. Arrows indicate the positions of mouse rRNA as molecular markers. RNA stained with acridine orange is shown in the bottom panel to assess the quality of RNA. (Right panels) Human Multiple Tissue Northern (MTN) blots (Clontech) containing 2 μg (left panel) or 1 μg (right panel) of poly A^+ RNA per lane. Blots were hybridized sequentially with human C5L2 (upper panel) or human C5a receptor (bottom panel) probes. Positions of RNA molecular markers are indicated. (D) Specificity of anti-C5L2. Flow cytometric analysis of L1.2 cells stably transfected with human C5L2 stained with rabbit anti-human C5L2, mouse anti-human C5aR, or an irrelevant antibody. (E) Human peripheral blood granulocytes stain positively for both C5L2 and C5aR.

Table 1: Binding Affinities for C5A and C5A des Arg on L1.2 Lymphoblasts, 293T Transfectants, or Peripheral Blood Leukocytes

A. 0.1 nM ^{125}I-C5a[a]

competing ligand cell type	apparent affinity nM ± SEM			
	C5a	C5a des Arg	C5a hexapaptide	C4a
C5L2/L1.2	2.5 ± 0.4 (7)	12.0 ± 2.3[b] (4)	18 ± 7 × 10^3 (3)	ND (3)
C5aR/L1.2	3.4 ± 0.7 (4)	660 ± 153[b] (4)	27 ± 9 × 10^3 (3)	ND (3)
human PMNs	4.5 ± 0.4 (3)	1900 ± 126[b] (4)	- -	- -

B. 0.5 nM ^{125}I-C3a[a]

competing ligand cell type	apparent affinity nM ± SEM
	C3a
C5L2/293T	ND (3)
C5aR/293T	ND (3)
C3aR/293T	5.1 ± 1.3 (4)

[a] (A) Cells were incubated with 0.1 nM ^{125}I-C5a and unlabeled C5a, C5a des Arg, C5a hexapeptide agonist, or C4a in competition binding assays or (B) with 0.5 nM ^{125}I-C3a and unlabeled C3a, as indicated in Experimental Procedures and apparent affinities (±SEM) calculated using PRISM software for n independent experiments. [b] Significantly different at $P < 0.005$. ND, not detected; - -, not done.

human PMNs for expression of the C5aR and C5L2 by two-color flow cytometric analyses. The representative results shown in Figure 1E clearly demonstrate coexpression of both receptors on essentially all cells in the monocyte/granulocyte pool.

C5L2 Is a High Affinity Receptor for Both C5a and C5a des Arg. As indicated above, because of the sequence similarity (~60%) and chromosomal localization to the C5aR/formyl peptide receptor cluster on human 19q13.4, we hypothesized that C5L2 may be a second C5a receptor. Importantly, previous studies using neutrophils in whole blood from the C5a receptor null mice demonstrated no G-protein mediated biological response following stimulation with C5a, in contrast to neutrophils from wild type animals (*12*). Thus, a positive result for binding C5a would be consistent with our prediction that C5L2 is a C5a binding protein that is not coupled to G proteins because of the mutations identified above.

Murine pre-B L1.2 cells were stably transfected with the human C5a receptor or with human C5L2 and studied in competition binding and signal transduction experiments. For some experiments, we used 293T cells transiently transfected with C5L2, C5aR, or C3aR with or without Ga16. C5aR/L1.2 cells bound C5a with an apparent K_d of 3.4 ± 0.7 nM ($n = 4$), consistent with previous reports (*1*). C5a des Arg competed with ^{125}I-C5a with approximately 200-fold less avidity, with a K_i of 660 ± 153 nM ($n = 4$) (Table 1 and Figure 2A). C5L2/L1.2 cells studied in parallel bound human C5a with an affinity almost identical to the C5a receptor (2.5 ± 0.4 nM, $n = 7$). In contrast to C5aR, C5L2/L1.2 cells exhibited an affinity for C5a des Arg almost as high as for C5a, at 12.0 ± 2.3 nM ($n = 4$). Thus, on identical cell types, C5L2 is approximately 50-fold more avid in binding C5a des Arg than the C5a receptor (Table 1 and Figure 2A,B). In contrast with previous reports (*25*, *26*), C4a did not compete with ^{125}I-C5a on C5L2/L1.2 cells (Figure 2B). Further, as shown in Table 1, when 293T cells were transiently transfected with C5L2, C5aR, or C3aR, only C3aR expressing cells exhibited binding of ^{125}I-C3a. When similar experiments were performed with human leukocytes, expressing both C5aR and C5L2, the apparent K_d for C5a was the same as on either L1.2 transfectant, but C5a des Arg was even less effective in competing for ^{125}I-C5a binding

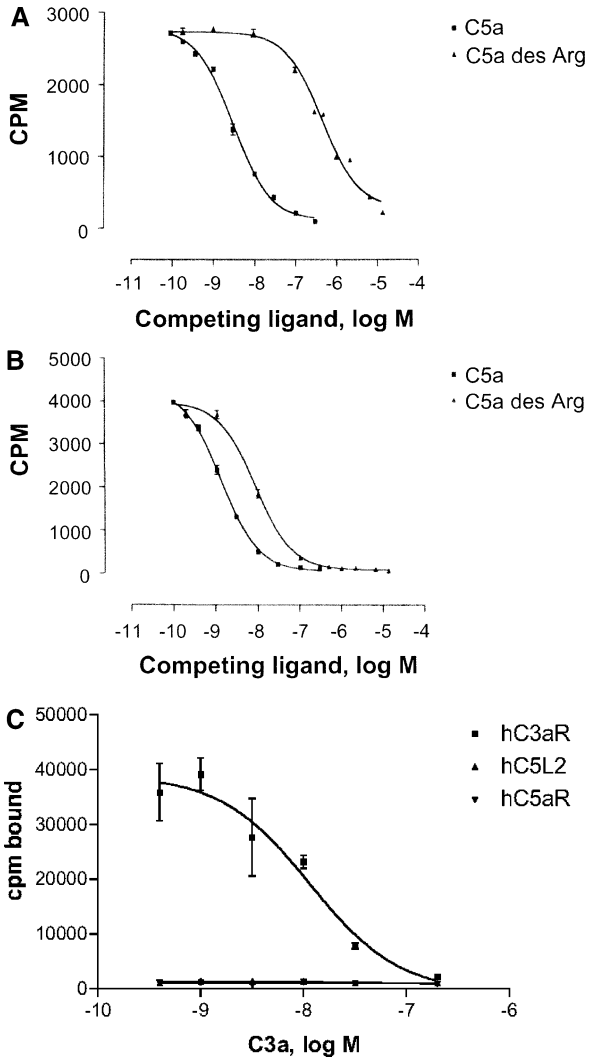

FIGURE 2: Ligand specificity of C5L2. Competition binding of L1.2 cells stably transfected with human C5aR (A) or C5L2 (B). Cells were incubated with 0.1 nM ^{125}I-C5a and increasing concentrations of C5a, C5a des Arg, or C4a as indicated. (C) Human peripheral blood PMNs incubated with 0.1 nM ^{125}I-C5a and unlabeled C5a or C5a des Arg. Data are expressed as % binding ± SEM and derived from at least three independent experiments.

than the C5aR alone, exhibiting an apparent K_i of 1.9 ± 0.1 μM ($n = 4$) (Table 1 and Figure 2C). These results suggest

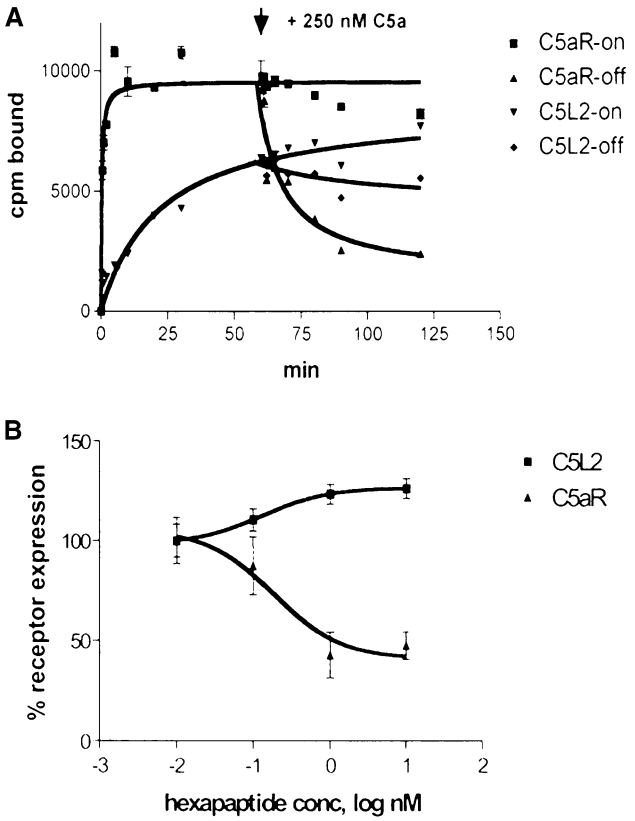

FIGURE 3: (A) Kinetics of binding C5a to the C5a receptor versus C5L2. L1.2 transfectants were incubated with 0.1 nM ^{125}I-C5a, and the ligand bound was assessed as a function of time. At 60 min, 250 nM unlabeled C5a was added to half the cells, and binding was assessed for an additional 60 min. (B) Internalization of C5aR and C5L2 on human PMNs. Neutrophils were incubated with 0−10 μM C5a hexapaptide for 5 min at 37 °C. Cells were subsequently transferred to ice, and receptor expression was evaluated by flow cytometric analyses.

a complex, potentially regulatory interplay between the C5aR and the C5L2 expressed on the same cell type.

Comparison of Scatchard analyses of sites per cell on L1.2 cells and the fluorescence intensity on both L1.2 cells and neutrophils suggests similar levels of expression of C5aR and C5L2 on this cell population. Further, analyses of the displacement binding curves by one- or two-site models provides no compelling evidence to distinguish the two; thus, the reduced affinity for C5a des Arg on PMNs is not as simple as the net effect of the presence of both receptors.

One possibility we considered was that since apparent affinities are determined by the ligand on- and off-rates, the differences between C5aR and C5L2 may reflect differences in binding kinetics. When the kinetics of C5a binding were assessed using L1.2 transfectants at 0 °C in the presence of sodium azide to prevent internalization, we observed that the C5aR has a very rapid on-rate, essentially at equilibrium within the first minutes following addition of ligand. In contrast, under identical conditions the on-rate for C5L2 was ~100-fold slower, requiring at least 60 min to reach equilibrium (Figure 3A). At equilibrium, addition of excess unlabeled C5a to displace the radioligand as an estimate of the off-rate revealed that the C5aR released ligand 3× faster than C5L2 (Figure 3A). Thus, under the conditions of binding to human neutrophils, the classical C5a receptor may be the only one detected.

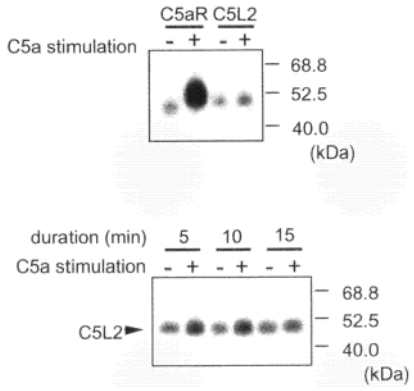

FIGURE 4: Receptor phosphorylation. Stable L1.2 transfectants were metabolically radiolabeled with [^{32}P]-orthophosphoric acid, stimulated with 0 or 200 nM C5a. Receptors were immunoprecipitated and analyzed by 10% SDS−PAGE. Cells were stimulated with C5a for 5 min (top) or as a function of time (bottom).

C5aR, But not C5L2, Is Rapidly Internalized Following Ligand Binding. A characteristic feature of G protein coupled receptors is their rapid internalization following the binding of ligands. We wanted to characterize this feature for C5L2 as compared with the C5aR. However, we previously showed the importance of the N-terminal sequence of the C5aR for binding ligand (*18*), and since our antisera are also directed against the N-terminal sequences of both receptors, we were concerned that bound C5a might interfere with the binding of antibody by steric competition, and this approach might not be useful to evaluate internalization. To circumvent this problem, we took advantage of a hexapeptide modeled on the C-terminus of C5a, which is a full agonist at the C5a receptor but interacts only with the pharmacophore site composed by the transmembrane helices and the juxtamembrane regions of the receptor (*21*). First, we demonstrated that the peptide was capable of competing for ^{125}I-C5a binding to both C5aR and C5L2 with similar affinities (Table 1). We then incubated human neutrophils with increasing concentrations of the hexapeptide for 5 min at 37 °C and examined cell surface expression of the receptors by flow cytometry. As shown in Figure 3B, the C5a receptor was rapidly internalized as a function of ligand concentration as expected, to a level of ~50% during 5 min, while the surface expression of C5L2 was virtually unchanged. Thus, C5L2 appears to be uncoupled to internalization in response to ligand.

Because the internalization of receptors has been shown to follow phosphorylation of serine and threonine residues in the C-terminal region (*27*), we determined whether C5aR and C5L2 are phosphorylated in response to ligand binding. Figure 4 shows the results of such an experiment. Both C5aR and C5L2 exhibit a basal level of phosphorylation in L1.2 transfectants in the absence of ligand. As indicated in Figure 4A, C5aR is robustly phosphorylated in response to 5 min exposure to agonist, while a very modest increase is seen for C5L2. As was previously noted, the mobility of the C5a receptor is slightly retarded after phosphorylation as well (*28*), while no change in mobility was observed for C5L2. As shown in Figure 4B, the ligand-dependent phosphorylation of C5L2 was maximal at 5−10 min and returned to baseline levels by 15 min.

Activation of the MAP Kinase Cascades by C5aR and C5L2. Activation of the MAP kinase family has been

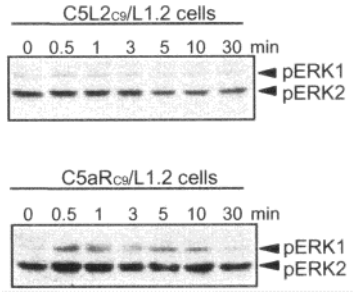

FIGURE 5: MAP kinase activation. Stable L1.2 transfectants were treated with 200 nM C5a for the indicated times. Cytosolic proteins were subjected to Western blot analyses for phosphorylated ERK1 and ERK2 as indicated. Phosphorylation was evaluated as a function of time (upper panels) or for the times indicated in duplicate in the presence or absence of C5a (lower panels).

demonstrated to occur for several G protein coupled receptors by a mechanism involving scaffolding of the phosphorylated receptors to arrestins, which may occur independently of and in parallel with activation of the G proteins (*27*). We were able to demonstrate activation of ERK-1 and ERK-2 by C5a only in cells transfected with the C5aR but not with cells expressing C5L2 (Figure 5). Thus, the relatively small extent of phosphorylation of C5L2 that occurs following exposure to C5a in these cells is apparently mirrored by minimal recognition by arrestins and subsequent activation of this transcription factor cascade.

C5L2 Is Obligately Uncoupled from G Proteins. The crystal structure for rhodopsin clearly demonstrates the essential nature of the guanidinium group of the arginine residue in the DRY sequence for formation of multiple hydrogen bonds with residues in transmembrane helices 3 and 6 (*29*). We and others have previously demonstrated that mutagenesis of this arginyl residue is associated with loss of function with respect to G protein activation, without alteration in ligand binding (*30*). We used this approach, for example, to dissociate G protein signaling from HIV-1 usage of CCR5 as a coreceptor for fusion and entry in cells (*31*).

To determine whether the natural substitution of leucine for arginine at this position was at least in part responsible for the failure of C5L2 to induce signal transduction, we tested the ability of C5a to induce changes in intracellular calcium concentration in these cells and observed no response in C5L2/L1.2 cells, while C5aR/L1.2 cells responded as previously observed (*30*). In transiently transfected human renal epithelial HEK293T cells, we previously demonstrated that coupling of C5a receptors to intracellular calcium flux is greatly facilitated by cotransfection with Gα16 (*24*). As shown in Figure 6, cells transfected with native C5L2 do not couple to calcium transients either in the presence or absence of Gα16. However, introduction of a point mutation in which leucine 132 is changed to arginine in this molecule is associated with a gain-of-function in coupling to Gα16. The response was significantly less robust than for cells transfected with wild type C5aR and Gα16 and did not decay in a manner similar to the wild type C5aR, likely reflecting the minimal ligand-dependent phosphorylation of C5L2. Thus, the conserved leucine at position 132, which exists in both human and murine C5L2, is a major factor leading to obligate uncoupling from G protein activation.

Microarray Analyses of C5aR and C5L2 Mediated Signal Transduction. Virtually all signal transduction leads, either

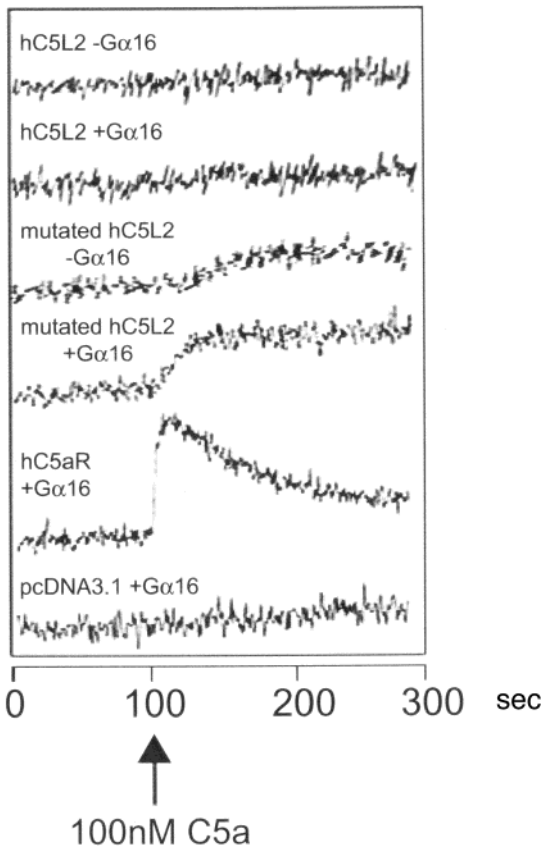

FIGURE 6: Increases in intracellular calcium by C5a. Fura-2 loaded 293T cells transfected with wild type C5L2, C5L2 L132R, or the C5aR, with or without Gα16, as indicated, were equilibrated at 37 °C. 100 nM C5a was added at the arrow, and increases in intracellular calcium were recorded as a function of time (s) by fluorescence.

directly or indirectly, to changes in mRNA expression patterns. We took advantage of the C5a receptor deficient mice to probe signaling by C5L2. Mouse bone marrow cells were incubated with 0 or 100 nM C5a, and after 4 h mRNA was prepared for microarray analyses using the mouse Affymetrix 10 000 gene chip. As shown in Table 2, wild type bone marrow, expressing both C5aR and C5L2, responded to C5a with reproducible patterns of up- and down-regulated messages. In the case of up-regulated genes, many are related to inflammation or its regulation. Strikingly, C5a led to a >20-fold induction in osteopontin-1, also known as early T cell activated gene-1 (ETA-1). In stark contrast, stimulation of bone marrow cells from the C5aR deficient mice, when only C5L2 is expressed, resulted in no detectable C5a mediated changes in gene expression. These data provide powerful evidence that C5L2 is a nonsignaling C5a/C5a des Arg binding protein.

DISCUSSION

C5a anaphylatoxin has been appreciated as a biological principal in serum since the early 1900s. The purification of C5a from complement activated sera from several species in the 1970s allowed for a much fuller characterization of its varied functions (*1*, *20*). In 1991, identification of the leukocyte receptor for C5a appeared to account for all of the known biological functions of the anaphylatoxin (*1*, *9*). However, some puzzling results lead to the possibility that other receptor(s) for C5a may exist. Differential effects of

Table 2: Changes in mRNA Expression in Wild Type (A) and C5aR-/- (B) Mouse Bone Marrow Cultures Following 4 h Stimulation with 100 nM Mouse C5a[a]

A. WT C5aR			
gene	fold induction	gene	fold suppression
X13986 osteopontin	20.60	L02914 aquaporin	2.73
A1323667 immunoregulatory gene 1	3.93	A18737110 mouse hypothalamus	2.42
L32838 mouse germline IL1 receptor antagonist	2.55	AF012104 BMX cytoplasmic tyrosine kinase	2.39
X13333 CD14	2.55	U20344 gut enriched Kruppel-like factor	2.30
X57437 histidine decarboxylase cluster	2.24		
Y07836 basic helix−loop−helix protein	2.16		
M62470 thrombospondin-1	2.15		
AA612450 mouse myotubes	2.13		
AV223216 type II IL1 receptor	2.12		
M34603 proteoglycan core protein	2.06		
AF061272 C-type lectin	2.03		
B. C5aR Knockout: No Genes Induced or Suppressed			

[a] Average of two separate experiments.

agonists and antagonists on leukocyte populations suggested the possibility of receptor(s) that may be either uniquely posttranslationally modified (*32*) or are perhaps coupled differently to intracellular signaling proteins (*33*, *34*).

Here, we show that a previously described orphan receptor, C5L2 (*17*), is in fact a second receptor for C5a. Unlike the classical C5aR, C5L2 is not coupled to intracellular G protein signaling pathways. C5L2 demonstrates several properties, in fact, that are consistent with a role as a decoy receptor. First, the receptor displays greatly retarded association kinetics with C5a, so that at low concentrations of C5a, such as might occur in a gradient in vivo, the classical C5aR would effectively sequester the available C5a. As higher concentrations of C5a are encountered, with the effect of reducing locomotion of the cells (chemotaxis curves are classically bell shaped), other C5a-dependent effects such as degranulation and local release of products of NADPH oxidase are elicited. Under these conditions, the C5L2 may effectively sequester C5a des Arg.

Alternatively, C5L2 may modulate the effective concentrations of C5a/C5a des Arg. Both C3a and C5a are rapidly degraded by serum carboxypeptidase N or carboxypeptidase R to their respective des Arg derivatives (*35*). At physiological concentrations, C3a des Arg is inactive at inducing smooth muscle contraction and increases in vascular permeability, whereas C5a des Arg retains approximately 10% of the activity of intact C5a. In a local setting where both C5a and C5a des Arg are present, with dynamically increasing quantities of C5a des Arg relative to C5a, C5L2 could serve to spare the C5a to interact with the functional receptor.

Another feature of C5L2 consistent with its role as a modulator of anaphylatoxin concentration is the fact that it has a very slow dissociation rate with respect to ligand and is effectively anchored at the cell surface. The classical C5aR binds C5a and is rapidly internalized. This has been demonstrated to result from phosphorylation of the C-terminus, a feature that is not significantly shared with C5L2. The functional C5aR is recycled to the cell surface while the ligand is degraded intracellularly. Thus, C5L2 could to be effective in clearing or limiting the inflammatory response to C5a, as a high affinity sink for ligand.

The concept of decoy receptors is well-established for the pro-inflammatory cytokines IL-1 and TNFα. Heretofore, however, the only example of a similar molecule in the family of G protein coupled receptors is the Duffy antigen receptor for chemokines (DARC) on RBC, which has significant affinity for certain chemokines (*36*). More recently, two additional members of the chemokine receptor family, D6 and CCRbp/CCR11 (*37*−*39*), have been shown to be high affinity binding receptors for β chemokines and CCR7/CCR9 ligands, respectively. Curiously, both of these receptors bear mutations in either TM2 or the DRY sequence that might be predicted to disrupt G protein coupling, as shown here for C5aR2. Also noteworthy is the observation that D6 is localized to lymphatic endothelium and may function to present chemokines to locally trafficking lymphocytes, macrophages, or dendritic cells (*40*). Similarly, the limited ligand binding profile of the CCRbp (effectively CCR7 and CCR9 ligands) may portend a role for this receptor in regulating trafficking of CCR7 and CCR9 positive cells, perhaps by sequestering or presenting ligands.

An unusual feature of the C5aR is that on neutrophils, effectively 100% of the receptor is precoupled to G proteins, with very little receptor present in the uncoupled lower affinity state. We could postulate that C5L2 somehow facilitates this feature, perhaps by heterodimerization. We acknowledge that our internalization experiments do not conclusively preclude formation of heterodimers. We have initiated targeted disruption of the C5L2 to address the role of this receptor in vivo.

While this manuscript was in preparation, two reports appeared that independently confirm our results with C5a and C5a des Arg binding to C5L2 (*25*, *26*). However, a number of significant differences exist between the overall results. In contrast with these reports, we do not observe interaction with C3a or C4a on this receptor, either in L1.2 lymphoblasts or 293T cells. In comparing our data with those reported (*25*), we can only note that the published binding isotherms are generated using ligand concentrations in a range only from 0.1 to 0.9 nM, which are inadequate as presented for mathematical conclusions. We also note that the number of receptor sites/cell in the case of C5a binding to stably transfected C5L2 in RBL cells was 39 736 + 5993. However, the values obtained for C3a showed 26 652 + 10 237. We suggest the latter standard error calls the data into question, especially when the specific binding is reported at 100−400 dpm. Consistent with these investigators, we do not observe significant signal transduction events associated with G protein activation pathways. In fact, we have demonstrated obligate uncoupling from G proteins that is

partially restored by mutating the L to R in the DRY sequence at the end of transmembrane segment three. 293T cells expressing this mutated receptor with Ga16 are partially restored in their ability to mediate ligand-induced changes in intracellular calcium concentration. The fluorescent decay characteristic of responses of the wild type C5aR is absent however, likely because of the relative lack of phosphorylation. Studies of C-terminally truncated G protein receptors, which are also not phosphorylated, demonstrate similar behavior (*41*). We have shown that C5a stimulates minimal activation of known distal pathways of signal transduction (such as MAP kinases) on C5L2 relative to the C5aR.

Cloning of the mouse C5L2, with demonstration of a similar replacement of arginine for leucine, suggests an essential function for this molecule among species. On human PMNs, C5L2 appears to be expressed at approximately equal numbers as the classical C5a receptor. Its slow kinetics of ligand association and dissociation relative to the classical C5aR, minimal phosphorylation in response to ligand binding, and lack of internalization of C5L2 on human neutrophils all point to a distinct role for C5L2. Additionally, we have performed microarray analyses demonstrating that C5L2 does not modify gene expression in the absence of the C5aR. Thus, at present we conclude that C5L2 likely functions as a regulator of C5a/C5a des Arg function, perhaps by modulating properties of the C5aR. Further information on the role of this receptor is being evaluated by targeted deletion of the gene from the mouse.

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